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THE DEVELOPMENT OF BREAST CANCER

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14. ABSTRACT  This project investigated the effects of rhythm disruption in mice on the expression of a wide range of genes in mammary tissue, the growth of MCF-7 xenografts and the growth, metastasis and gene expression in spontaneous mouse mammary tumors.  Subjecting mice to a simulated shiftwork schedule altered the pattern and level of expression of clock gene transcription factors and genes that are involved in the cell cycle. We have also found that the formation of secondary lung tumors is enhanced by a simulated shiftwork protocol.					
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## INTRODUCTION

Economic globalization has driven a high proportion of the population to engage in shiftwork. While this practice is not intrinsically detrimental, there is a growing body of evidence suggesting that rotational shiftwork rosters that result in disruption of circadian homeostasis have a negative impact on many aspects of human health (Megdal et al., 2005). The World Health Organization has now declared shiftwork to be a probable carcinogen by placing it in category 2A of agents likely to produce cancer based on the results obtained in a wide range of systematic reviews (Straif et al., 2007). In particular some epidemiological studies have shown up to a 48% increased risk of breast cancer in female shiftworkers. This association between cancer and shiftwork is likely to be due to a combination of lifestyle factors that directly or indirectly promote oncogenesis. To further understand this putative cause-effect relationship our laboratory has investigated how disruption of the circadian timing system affects the molecular processes that underlie the genesis and progression of breast cancer.

Few, if any physiological processes in the body escape regulation by the circadian system. It is now well accepted that every cell in the body possesses a molecular, cell-autonomous, time-keeping system that is driven by complex feedback loops within each cell. In the context of the organism's physiological homeostasis, the individual cellular oscillators are tightly synchronised at an organ-specific level and are kept in synchrony with the external environment through neural and humoral signals that originate in the suprachiasmatic nuclei (hypothalamus), which are susceptible to entrainment by external environmental cues such as the light-dark cycle (Reppert and Weaver, 2002)

At the cellular level a suite of core clock genes make up the positive (*Bmal1*, *Clock*,) and negative (*Per1*, *Per2*, *Cry1* and *Cry2*) driving forces in these feedback loops whose cycling unfolds over a period of approximately 24 hours. *Reverb-α* also plays a regulatory role in the cycling of the cellular clock. Briefly, the protein product of *Bmal1* dimerizes with CLOCK and drives the transcription of the *Per*'s and *Cry*'s by binding to E-box elements in the promoter region of these genes. The BMAL1:CLOCK dimer is also capable of regulating the expression of a wide range of other genes in the cell through the same mechanism. Indeed, several microarray studies have examined 24-hour variation patterns of gene expression and suggest that up to 10% of the transcriptome is controlled by the circadian time-keeping system (Akhtar et al., 2002). Amongst these genes there are important components and regulators of the cell cycle, which are the focus of the current project. For example, we know that *Wee1* or *cMyc* have regulatory E-box sequences in their promoter region (Fu et al., 2002, Matsuo et al., 2003). Apart from this, mouse knockout models have shown that genes from the *Per* family act as tumor suppressors although the mechanism of this is not fully understood. Finally, *Tim*, a somewhat controversial core-clock gene (Barnes et al., 2003) plays an important role in the regulation of the DNA damage pathway.

There is extensive evidence that maximal rates of cell division occur during certain times of the day (Matsuo et al., 2003). Because the main hallmark of cancer is abnormal cell division it is important to gain a full understanding of how the circadian clock regulates cell division and how disruption of the clock affects this process. Examining the role of the circadian system in the regulation of cell division and apoptosis is especially important.

## Body

### **General hypothesis:**

**Shiftwork and disrupted peripheral tissue rhythmicity facilitates the development of breast cancer.**

### **Specific hypothesis:**

**Disruption of clock gene rhythmicity alters the expression of oncogenes making mammary cells susceptible to spontaneous transformation into cancer cells and/or altering the rate of growth of tumors.**

**Mismatching of the photoperiod environment and nutrient intake and subsequent altered glucose, lipid and glucocorticoid levels, alters the expression of clock genes in mammary tissue and this leads to altered expression of oncogenes and other genes involved in the regulation of cell division.**

To test this hypothesis several new models were used that are designed to mimic the shiftwork situation as well as existing models, for example, the Polyoma Middle T oncoprotein mouse (PyMT) and xenografts of MCF-7 cells in immunodeficient mice.

### **Task 1 Expression of clock and clock controlled genes in mammary tissue of mice under simulated shift work conditions (rapid phase shifting of LD cycle).**

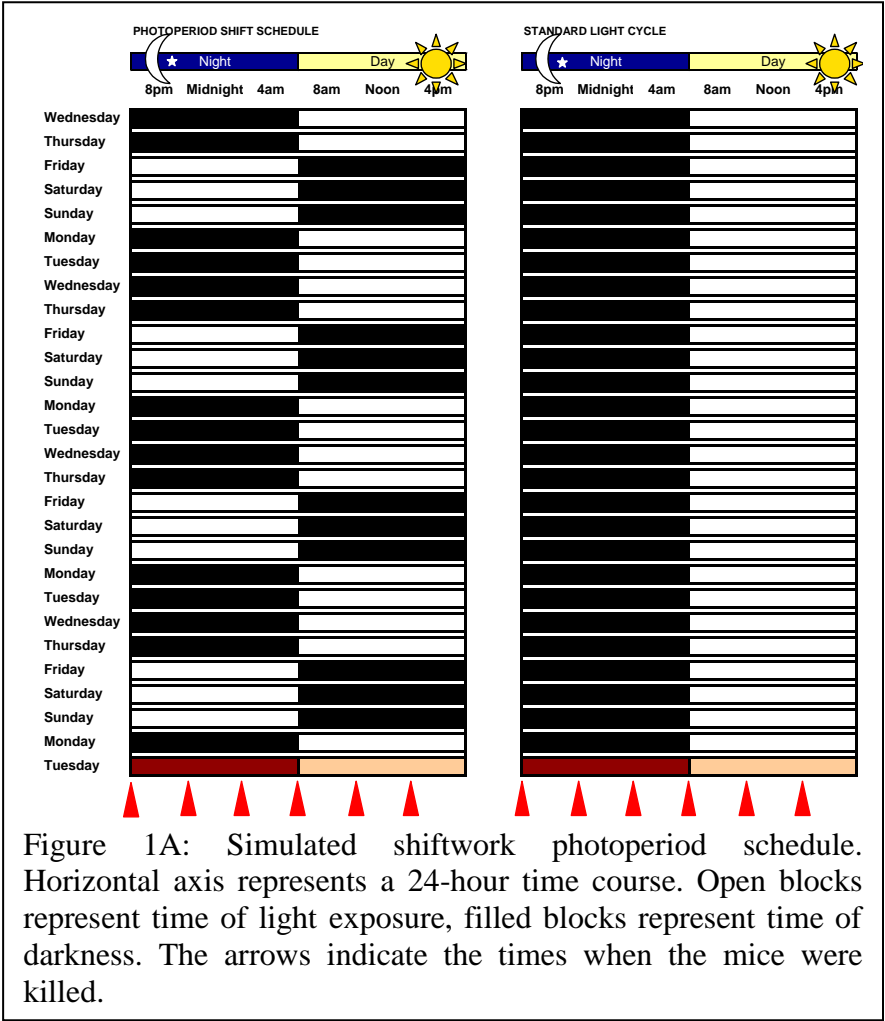
**Aim:** To determine the impact of rapid phase shifting of the light dark cycle on clock and clock controlled gene expression in the mouse.

In this experiment the expression of a wide variety of core-clock and cell-cycle genes was measured over a 24 hour period and the impact of a simulated shiftwork protocol on the expression pattern of these genes was investigated.

Sixty adult female BALB/c mice were housed in groups of 5 in 12 individual cages. Temperature was constant at 22°C and food and water were available *ad libitum*. All animals were kept in ventilated light tight boxes: a) 6 cages comprising the shiftwork animals had their 12L:12D photoperiod inverted for 3 consecutive 24-hour periods every week for a total of 4 weeks, b) 6 cages acted as a control and were kept in an identical light tight box with 12L:12D lighting schedule throughout the entire experimental period (Fig 1A).

Twenty four hours after the first night of the final reversal to non-shiftwork lighting conditions, 5 animals from each group (shiftwork and control) were killed by cervical dislocation at CT12 (12 hours after lights on, ie 1900h) and 4-hourly thereafter over a 24 hour period.

The 4<sup>th</sup> inguinal mammary pad was immediately removed, immersed in RNALater<sup>®</sup> and kept at -20°C until processed. Mammary tissue was homogenized and the RNA extracted using a Qiagen<sup>®</sup> Lipid Extraction kit followed by DNase digestion. Quantity and quality of RNA was assessed using a Eppendorf<sup>®</sup> Spectrophotometer and 2 µg of RNA were reverse-transcribed using Superscript III<sup>®</sup> kits (Invitrogen<sup>®</sup>). Results from Realtime PCR were analysed using the  $2^{-\Delta\Delta CT}$  method with  $\beta$ -actin as a housekeeper gene. For any cycle thresholds that were greater than 31 cycles, the expression was considered to be less than the detection limit and for statistical purposes allocated a value of 31.



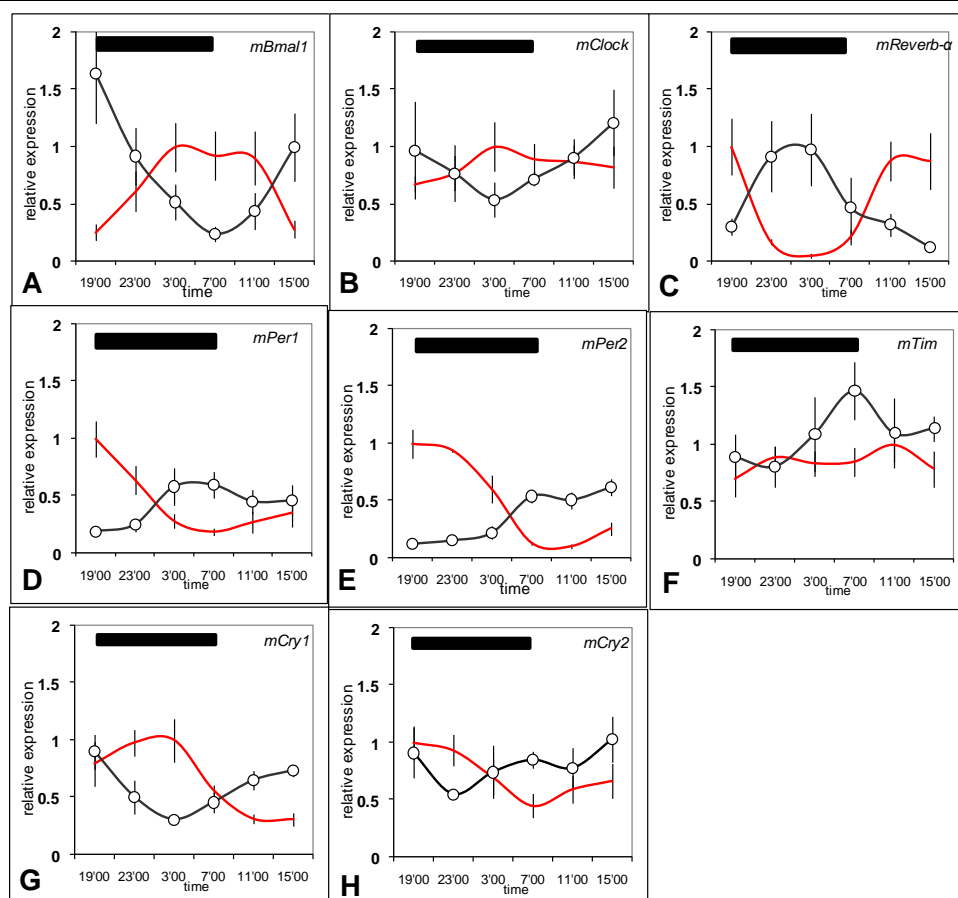
## Results

### a) Effect of shiftwork on the expression of core clock genes in mouse mammary tissue

The circadian rhythmicity of mammary tissue gene expression under normal and simulated shiftwork conditions was assessed. Figure 1B shows the 24 hour expression profile of 8 core clock genes in mammary tissue. All of the core clock genes were rhythmically expressed in mice subjected to a normal 12L:12D photoperiod except for *mTim* and *mClock*, as determined by Cosinor analysis ( $p < 0.05$ ). This finding is in accordance with previous information published in the literature (Metz et al., 2006).

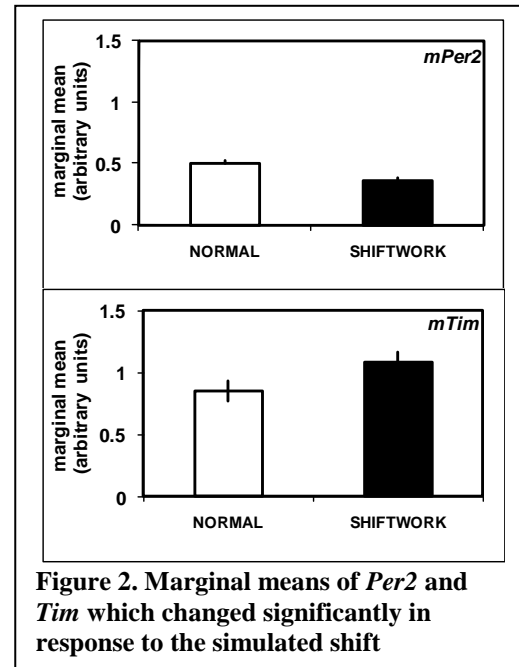
Simulated shiftwork caused a loss of circadian rhythmicity in *mCry2* and *mPer2* expression one day after the last change back to the normal photoperiod (Cosinor,  $p < 0.05$ ). While all other clock genes examined retained rhythmicity, the pattern was in antiphase to the control group (Levi et al., 1995).

In addition to the altered circadian rhythm of expression, there was a significant down-regulation of *mPer2* (29%) and an up-regulation of *mTim* (22%) over the 24 hour period ( $p < 0.05$ ) (fig 2). 2-way ANOVA indicated significant time of day X experimental treatment interactions in *mBmal1*, *mCry1*, *mPer2*, *mPer1*, *mRev erba*. and *mP21* mRNA expression.



**Figure 1B.-** Twenty four hour expression profile of core clock gene expression in the mice subjected to a 12L:12D photoperiod (no symbols) or to a shiftwork simulation (open circles), one day after the last change back to the normal photoperiod. The peak expression of the control mice was set at 1. Data are the mean  $\pm$  SEM (n=4-5).

Shiftwork has a major impact on the expression pattern of clock genes in mammary tissue. It was predicted that the animals subjected to the simulated shiftwork schedule would have a dampened level of expression throughout the 24 hour period. Figure 1 shows that instead the shiftwork animals had a 24 hour expression profile which was in antiphase with respect to the animals on a 12L:12D photoperiod. The consequence is that rhythmic gene expression was desynchronised from behavioural (activity/feeding) rhythm of these animals. Undoubtedly, this altered pattern of expression of core-clock genes will be reflected in those genes and processes that are downstream of the circadian clock. A particularly important finding was that the *mPer2* mRNA expression was globally downregulated during the 24 hours of evaluation. PER2 is implicated in the cell cycle and mice deficient in this protein develop tumors more readily than wild-type mice (Fu et al., 2002). This provides further circumstantial evidence for the hypothesized negative impact of shiftwork on the incidence of breast cancer.



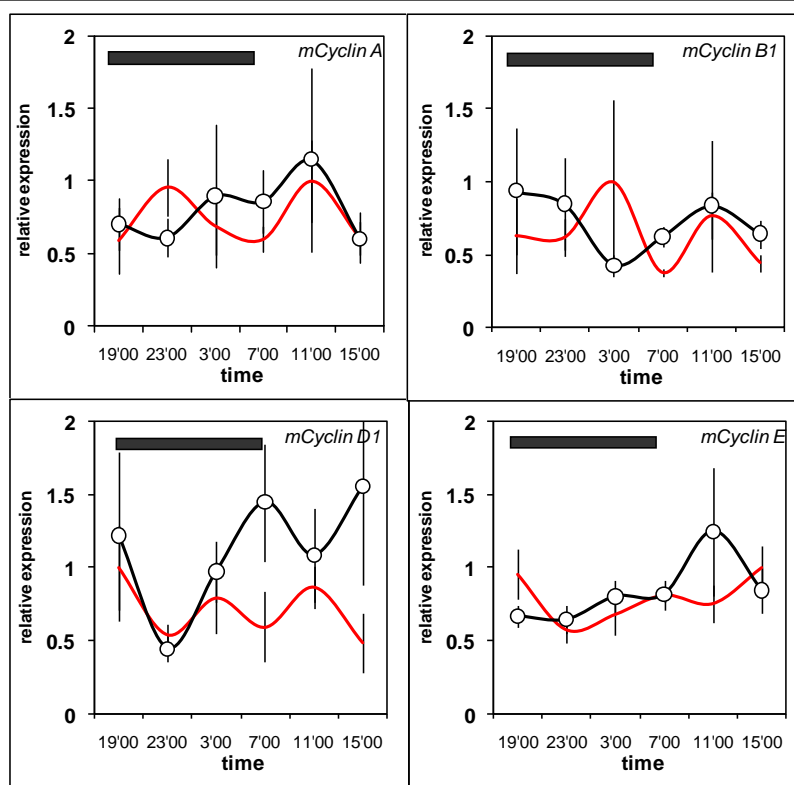
**Figure 2. Marginal means of *Per2* and *Tim* which changed significantly in response to the simulated shift**

**b) Effect of shiftwork on the expression of cell cycle genes in mouse mammary tissue**

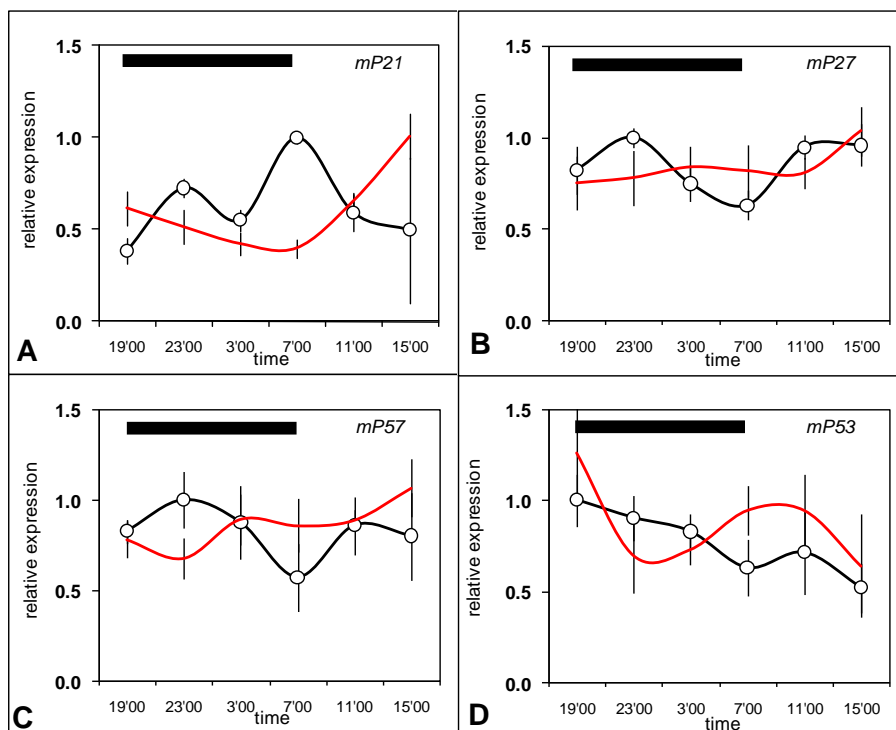
The expression patterns of *Cyclin A*, *B1*, *D1* and *E* mRNA were studied in the mammary glands of the mice. In contrast to the report by Fu and co-workers (Fu et al., 2002), none of the *Cyclin* genes were expressed rhythmically in animals under a 12L:12D photoperiod (fig 3). This difference may be due to strain differences (Balb/c in the current study vs. 129/C57Bl6 for Fu et al., 2002). The simulated shiftwork did not alter the expression of *Cyclin A*, *B1*, *D1* or *E* mRNA. The expression of other genes involved in the cell cycle and in the DNA-damage pathways were also studied in these mice. Expression of *mP21* (fig. 4), *mChk1* (fig. 5), *mWee1* (fig 6) and *mTgfb* (fig. 7) varied significantly with time ( $p < 0.05$ ). Expression of *Chk1* and *Tgfb* did not change with time in mice subjected to the simulated shiftwork and the overall expression was not affected by shiftwork in either gene. There are previous reports showing that *Wee1* and *Tgfb* are rhythmically expressed in a 12L:12D photoperiod in liver, but to our knowledge this is the first time a circadian rhythm of expression has been described for *P21* or *Chk1*. Both of the latter genes are involved in the DNA damage pathway and are capable of stalling the cell cycle. It is difficult, however to speculate on what the functional consequences for the cell cycle of the ecto-chronologic expression of these genes. We have attempted to identify some of the functional consequences of these changes in the experiments described below. Interestingly expression of *mATM* mRNA was up-regulated by 33% (fig. 8) in mice subjected to the shiftwork simulation. Expression of *Brca1*, *CDC25*, *Chk2*, *c-myc*, *gadd45a*, *P27*, *P53* and, *P57* mRNA was neither rhythmic, nor altered following the shiftwork simulation.

ATM	CHK1	CYCLIN A	CYCLIN E	P27	TGFB
BRCA1	CHK2	CYCLINB1	GADD45a	P53	WEE1
CDC25	cMYC	CYCLIND1	P21	P57	

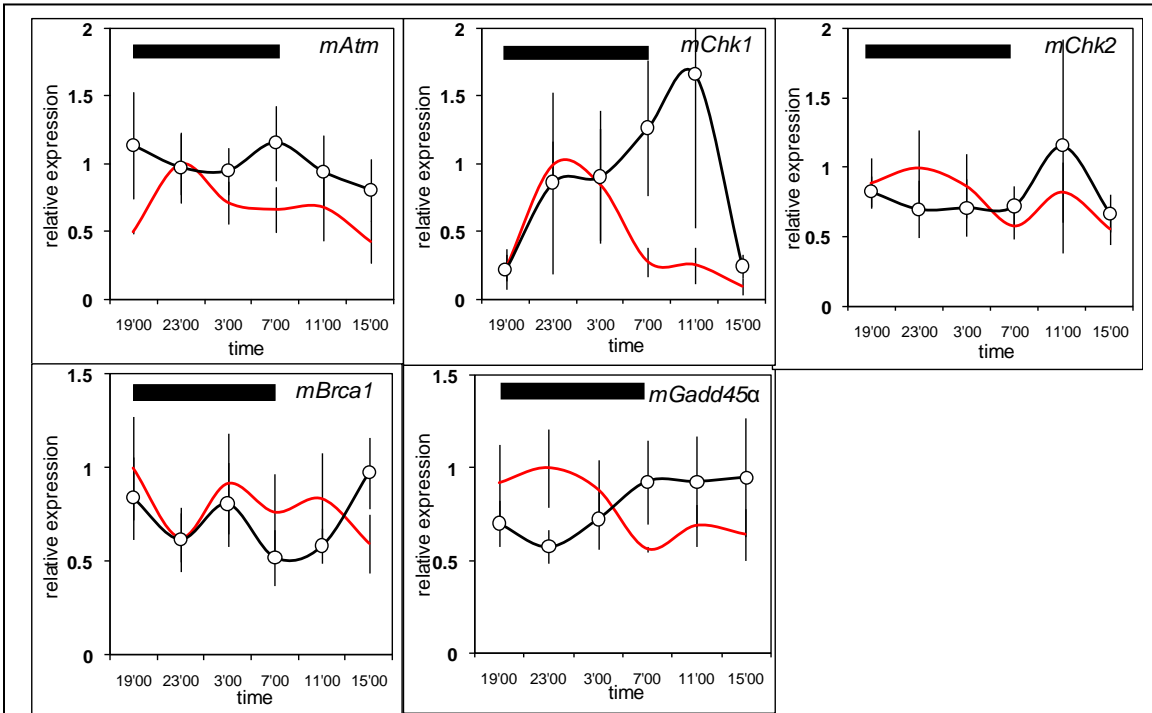
**Table 1.- List of cell cycle genes examined in this experiment**



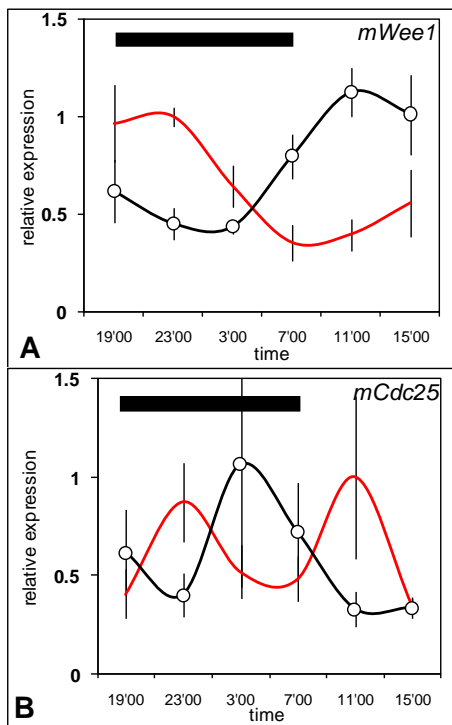
**Figure 3** 24 hour profile of *Cyclin A, B1, D1 & E* mRNA expression in mammary tissue of mice under a 12L:12D photoperiod (no symbols) or after exposure to a simulated shift work schedule (open circles).



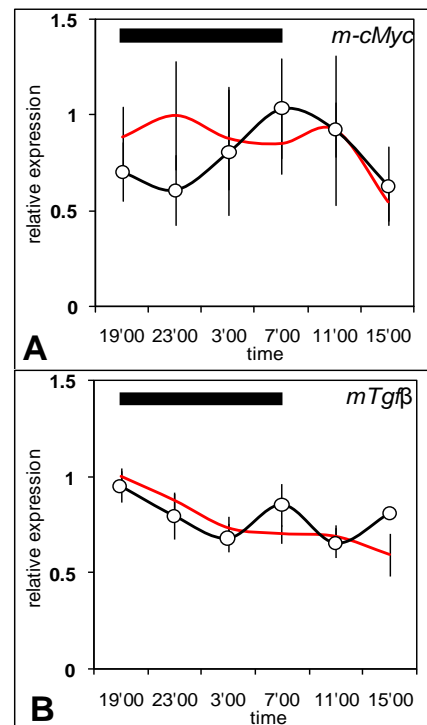
**Figure 4.-** 24-hour profile of *P21, P27, P57 & P53* mRNA expression in mammary tissue of mice under a 12L:12D photoperiod (no symbols) or after exposure to a simulated shift work schedule (open circles).



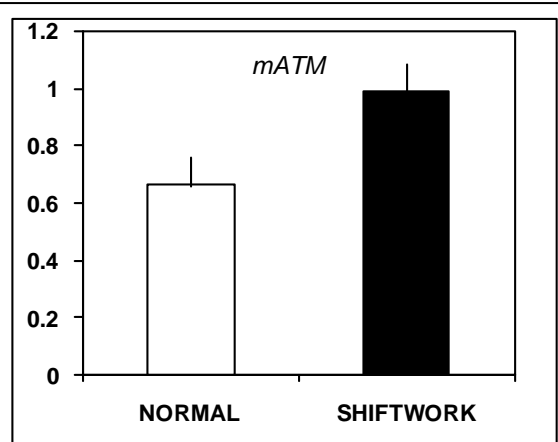
**Figure 5.-** 24-hour profile of *Atm*, *Chk1*, *Chk2*, *Brca1* & *Gadd45a* mRNA expression in mammary tissue of mice under a 12L:12D photoperiod (no symbols) or after exposure to a simulated shift work schedule (open circles).



**Figure 6.-** 24-hour profile of *Wee1* & *Cdc25* mRNA expression in mammary tissue of mice under a 12L:12D photoperiod (no symbols) or after exposure to a simulated shift work schedule (open circles).



**Figure 7.-** 24-hour profile of *c-Myc* & *Tgfβ* mRNA expression in mammary tissue of mice under a 12L:12D photoperiod (no symbols) or after exposure to a simulated shift work schedule (open circles).



**Figure 8.- Simulated shiftwork produced a global increase in the level of expression of *mAtm* when compared to mice under a 12L:12D photoperiod(white).**

## **Task 2 Growth and gene expression of human breast cancer cells in nude BALB/c-Foxn1<sup>nu</sup> mice under simulated shift work conditions**

SCID<sup>1</sup> mice were subjected to the shiftwork schedule for 4 weeks prior to the subcutaneous injection of a suspension of MCF-7 cells ( $5 \times 10^6$ ; in Matrigel® Becton Dickson). Tumor volume was measured at 2, 3 and 4 weeks after implantation (mice remained in the shiftwork simulation during this period). After 4 weeks the mice were sacrificed at 4 hourly intervals over a period of 24 hours and the tumors, liver and mammary glands were collected in RNALater® (Ambion) for gene analysis.

Several technical difficulties were experienced during this experiment. In a first experiment, the majority of the mice became unwell and had to be euthanized. After obtaining veterinary advice we were advised that genital infections are a common occurrence when estrogenizing immunodeficient mice; a necessary pre-requisite when growing estrogen-dependent cell lines as xenografts. In a subsequent experiment BALB/c-Foxn1<sup>nu</sup> mice were used and antibiotics added to the drinking water throughout the experiment. It is interesting that in the first experiment most of the sick animals were in the simulated shiftwork group, thus suggesting that the continuous shifting may have exacerbated the negative effects of estrogenization. This observation certainly deserves further examination in an attempt to understand the mechanisms that underlie this higher mortality.

### **Results**

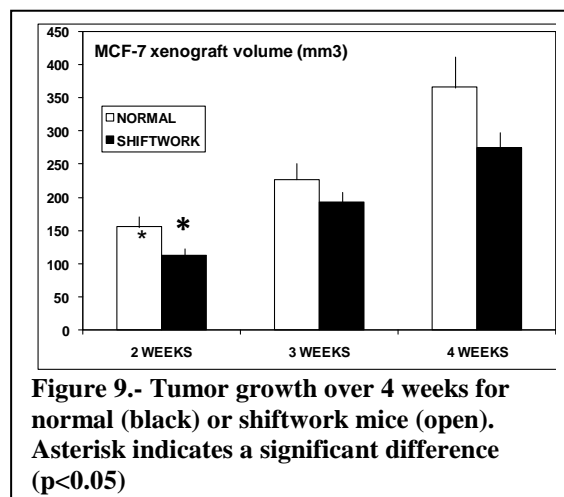
#### **a) Effect of shift work on tumor growth rate**

There was a significant difference in tumor volume after 2 weeks with a lower volume in the animals under the shiftwork simulation. A trend towards lower tumor volumes persisted in the shiftwork mice, but no significant differences were observed throughout the remainder of the experiment (fig. 9).

#### **b) Effect of shiftwork on clock genes in mammary tissue of nude BALB/c-Foxn1<sup>nu</sup> mice**

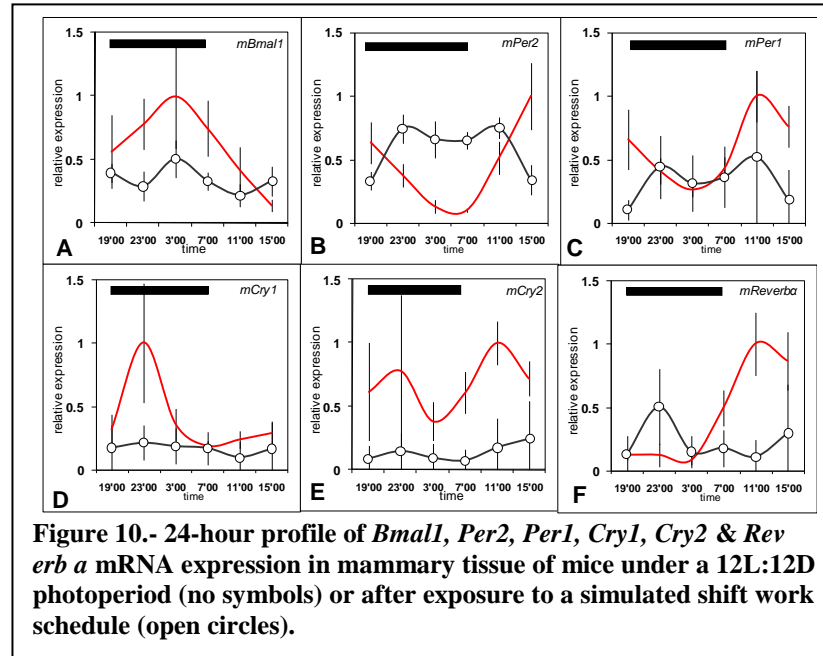
The 24-hour expression profile of 6 clock genes in the mammary tissue of nude BALB/c-Foxn1<sup>nu</sup> mice was determined in animals kept in 12L:12D or 1 day after returning to the normal photoperiod after 8 weeks of shiftwork simulation (fig. 10). Expression of *mBmal1*, *mPer1* and *mRev erba* was rhythmic in the mice kept on the 12L:12D photoperiod. Expression of *mCry1* or *mCry2* did not change significantly across 24 hours.

The simulated shiftwork protocol caused the circadian rhythm of expression of *mBmal1*, *mPer1*, *mPer2* and *mRev erba* to be lost (fig. 10). Furthermore, the expression *mBmal1*, *mPer1*, *mCry1*, *mCry2* and *mRev erba* were all significantly down-regulated in the mice subjected to shiftwork compared to those kept under the invariable 12L:12D photoperiod (see fig 11).

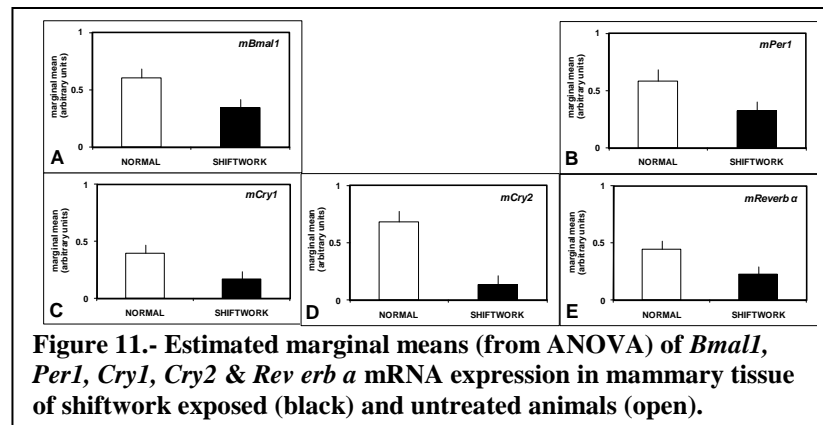


<sup>1</sup> SCID mice instead of nude mice were used in this experiment as these were found to be more resilient than nude mice.

The functional cellular timing system in the mammary gland was disrupted by 8 weeks of simulated shiftwork. The results are in contrast to the gene profiles obtained in the mammary tissue of Balb/c mice kept in these conditions for 4 weeks. In this experiment the profiles of gene expression following the shiftwork simulation do not appear in antiphase but were dampened.



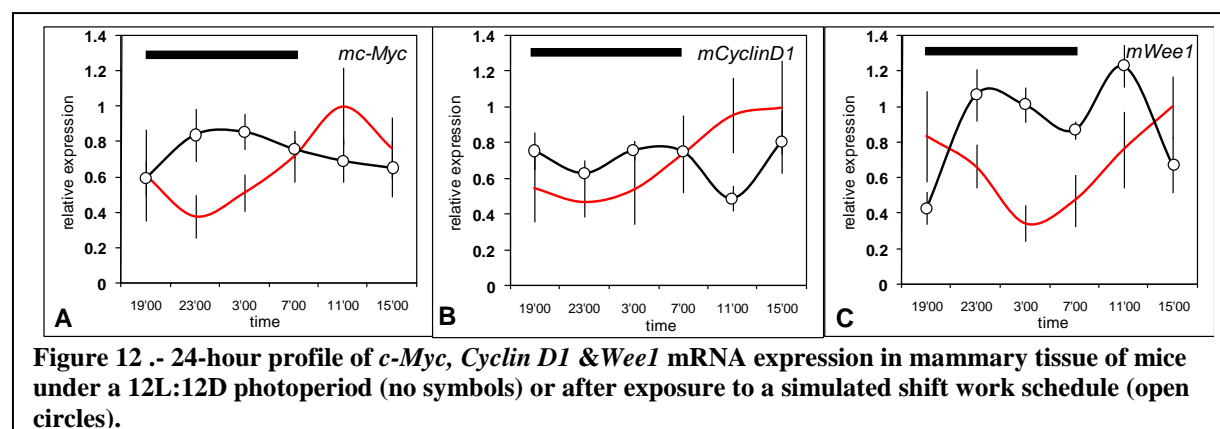
**Figure 10.- 24-hour profile of *Bmal1*, *Per2*, *Per1*, *Cry1*, *Cry2* & *Rev erba* mRNA expression in mammary tissue of mice under a 12L:12D photoperiod (no symbols) or after exposure to a simulated shift work schedule (open circles).**



**Figure 11.- Estimated marginal means (from ANOVA) of *Bmal1*, *Per1*, *Cry1*, *Cry2* & *Rev erba* mRNA expression in mammary tissue of shiftwork exposed (black) and untreated animals (open).**

### c) Effect of shiftwork on cell cycle genes in mammary tissue

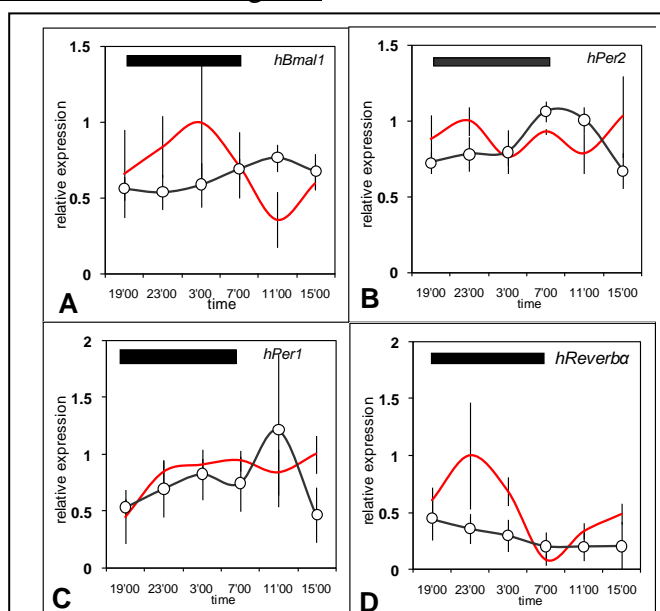
Expression of *m-cMyc* mRNA was rhythmic in the mammary tissue under normal 12L:12D photoperiodic conditions (fig. 12). This is in contrast with the pattern of expression of this gene in the mammary tissue of normal (immunocompetent) BALB/c mice in the previous experiment (see fig 7). Another difference with respect to the previous experiment is that *mWee1* mRNA expression showed no significant rhythmicity in mammary tissue by Cosinor Analysis in nude mice, although there was a trend for lower expression during the late dark period similar to that shown in figure 6. Expression of *cyclin D1* was neither rhythmically expressed nor affected by the shiftwork simulation.



### d) Effect of shiftwork on clock gene expression in MCF-7 xenografts

The 24-hour expression profile of *hBmal1*, *hPer1*, *hPer2* and *hRev erba* mRNA was examined in the MCF-7 xenografts to determine if the host circadian timing regulated gene expression in the tumors. All 4 genes were expressed in the tumors (fig. 13) but no rhythmicity was detected by Cosinor analysis. There was, however, a trend towards a higher expression of *hBmal1* in the 2<sup>nd</sup> half of the night, while *hRev erba* appeared to be expressed at higher levels in the first half of the night. The simulated shiftwork protocol eliminated these trends.

There was a significant decrease in the global level of expression of *hRev erba* in the xenografts of shiftwork mice compared to the controls (47%).

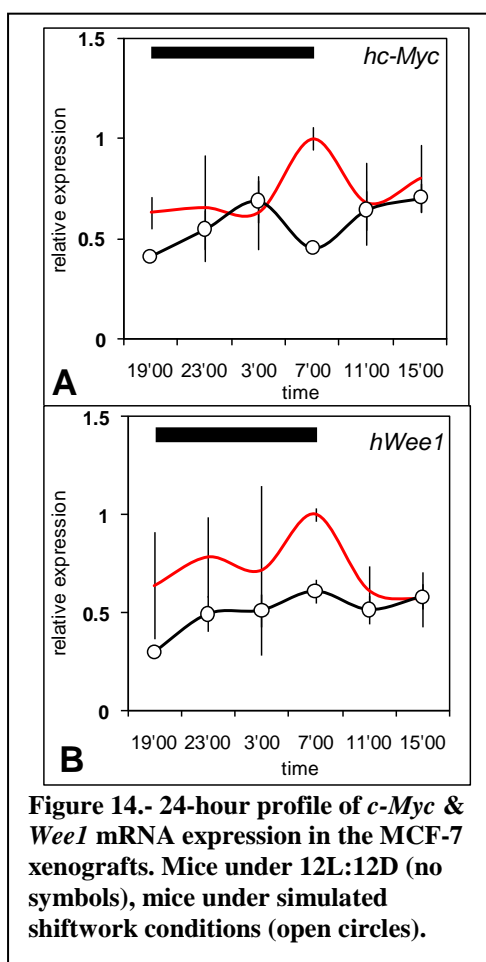


### e) Effect of shiftwork simulation on cell cycle genes in MCF-7 xenografts

This part of the project produced some difficult technical challenges that have been only partially resolved. Clock genes and cell-cycle genes are very well conserved throughout evolution and it is extremely challenging to design primers that are species-specific. To date it has been possible to design specific primers only for *h-cMyc* and *hWee1* to assess the 24-hour gene expression profiles in the human parenchyma of the xenografts and not in the murine stroma that is supporting these cells. Neither of these genes displayed circadian rhythmicity as determined by Cosinor analysis. Exposure to the shiftwork simulation did not affect the global level of expression of either of these genes.

We have shown (fig. 14) that *h-cMyc* and *hWee1* do not show circadian rhythmicity in their 24-hour expression profile and that the shiftwork protocol does not have a strong impact on the pattern of expression of these 2 genes.

In conclusion, it would appear that the host's circadian clock does not have an effect on the pattern of gene expression within the xenograft. This statement is based on the fact that we know *Wee1* is under direct control of the circadian system under normal conditions given that it contains an element that is responsive to core clock genes within its promoter region.



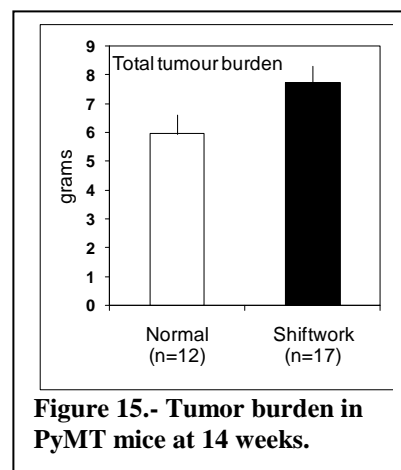
### **Task 3 Growth and progression to malignancy of mammary tumors in PyMT transgenic mice under simulated shift work conditions**

The purpose of this experiment was to determine the effects of shiftwork on the rate of tumor growth and the 24 hour expression profile of core-clock genes in the and cell-cycle genes in the tumors PyMT transgenic mice (Lin et al., 2003).

Briefly, heterozygous PyMT female mice were subjected to the shiftwork schedule or kept under 12L:12 from weaning (3 weeks) until the age of 14 weeks. At this time 4-5 animals were killed by cervical dislocation 4-hourly over a 24-hour period. The primary tumor of the 4<sup>th</sup> inguinal fat pad was dissected and a portion stored in RNA-Later<sup>®</sup> (Ambion) for subsequent analysis. Another sample of the same tumor was snap frozen in embedding medium (Tissue-tech<sup>®</sup>) for later analysis. Finally, the entire tumor mass of the animal was removed and weighed to determine the tumor burden.

#### **a) Tumor growth:**

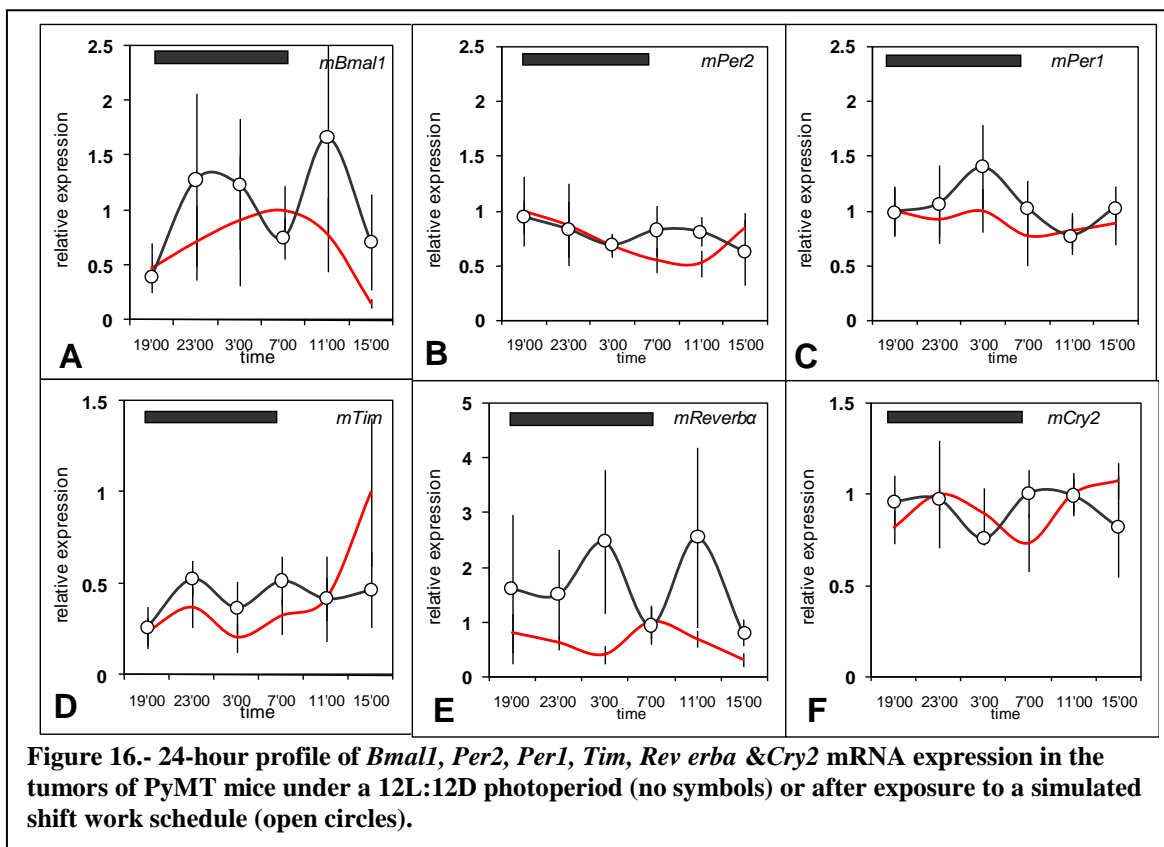
The tumor burden of the animals subjected to the shiftwork simulation was significantly greater than in the controls ( $p < 0.03$ , one tailed; fig. 15).



**b) Effect of shiftwork on the expression of core clock genes in tumors of PyMT mice:**

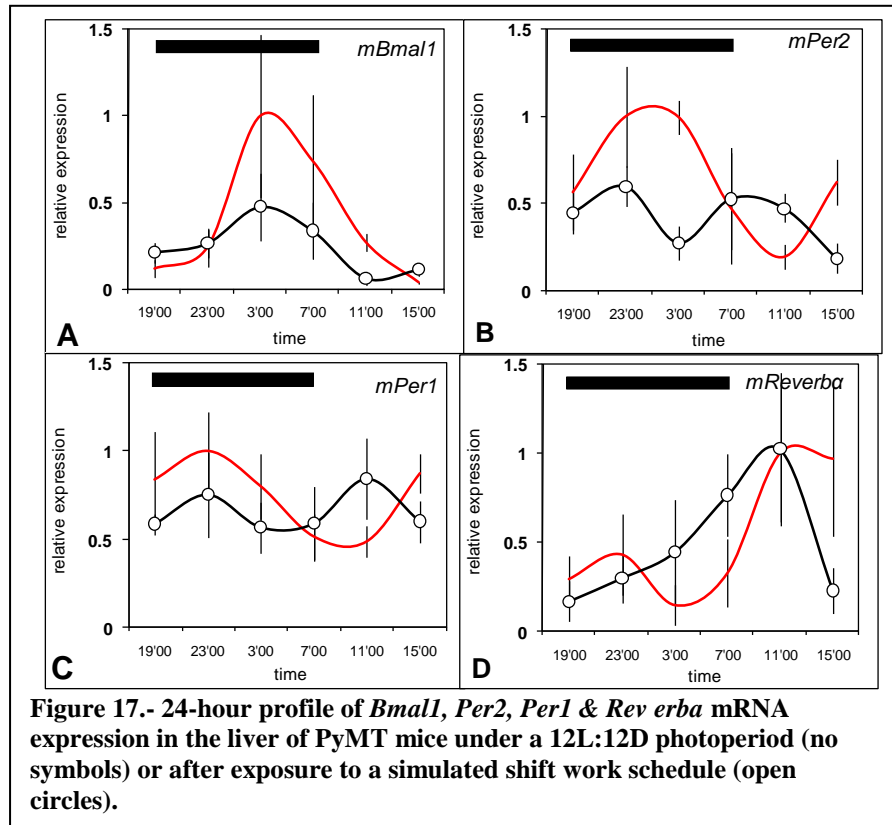
Expression of *mBmal1*, *mPer1*, *mPer2*, *mRev erba*, *mTim* and *mCry2* was measured in the tumor tissue of the PyMT mice (fig. 16). Cosinor analysis of the 24-hour pattern of gene expression did not reveal a circadian rhythm for any of the clock genes of tissue taken from the mammary tumors in either of the treatments. While there is no rhythmicity it is important to take into account the phase relationships of the different peaks of expression of the core clock genes. The fact that *mBmal1* and *mPer2* appear in antiphase suggests that there may be at least a partially functional time-keeping mechanism in these tumors.

It is known from the two earlier experiments described above that normal mammary tissue has a functional clock that is disrupted or dramatically shifted when the mice are subjected to a simulated shiftwork schedule. Consequently, it would be interesting in future experiments to verify if there is a functional clock in the normal mammary tissue of the wild-type mice lacking the PyMT transgene. While the variability is quite large, it would appear that shiftwork has a higher impact on *mBmal1* and *mRev erba*, displaying a bi-modal profile for these two genes, which is similar to the profile displayed by some of the cell cycle genes, as shown below. Whether this is an indication of interaction amongst these genes is a matter that will require future investigation.



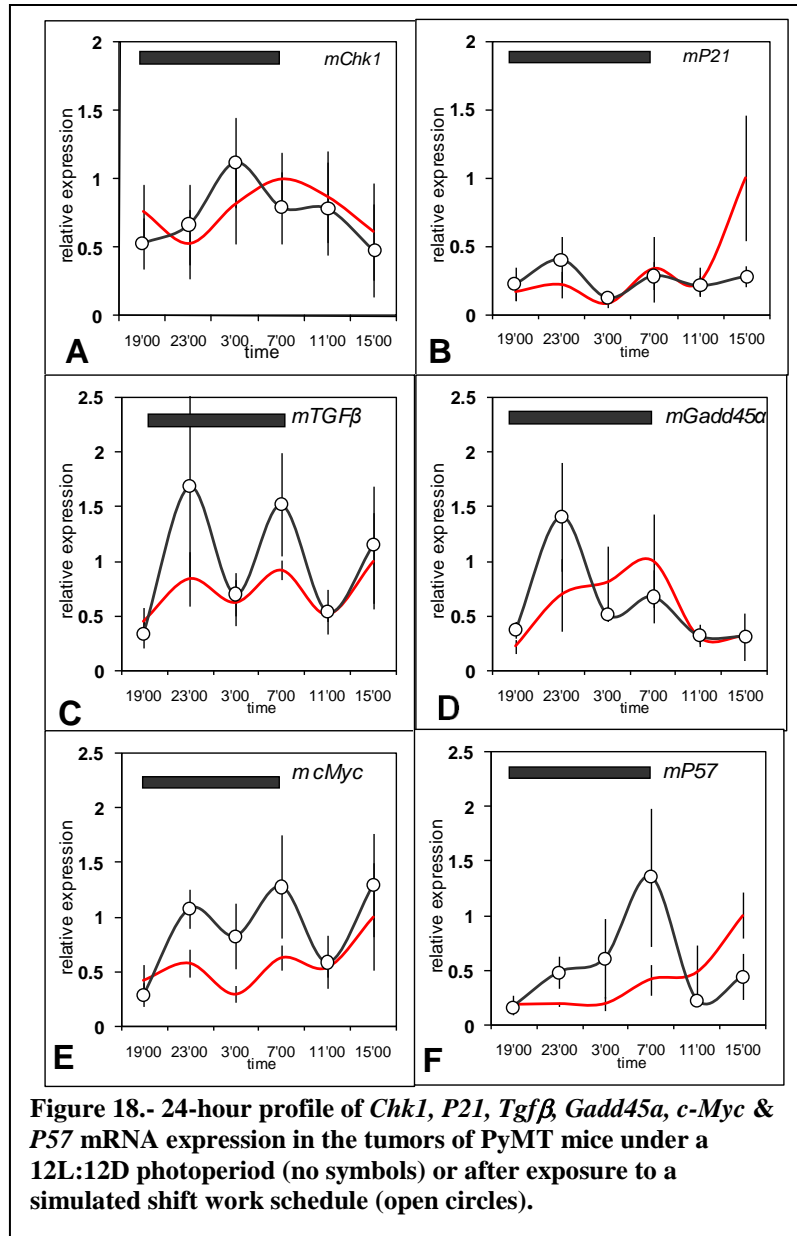
### c) Effect of shiftwork on the expression of core clock genes in the liver of PyMT mice

The expression of *mBmal1*, *mPer1*, *mPer2* and *mRev erba* mRNA in the liver of PyMT mice was measured to gain an insight into the rhythmic status of the animals since the background strain (FvB) was different from the strains used elsewhere in this study. As expected rhythms of expression were detected in all 4 genes and their timing was consistent with a functional circadian timing system (fig. 17). The amplitude of the rhythmic expression of *mPer2* and *mBmal1* was dampened following exposure to the shiftwork simulation for 11 weeks. There was a global decrease in the level of expression of *mPer2*, a common observation in previous experiments following the shiftwork simulation.



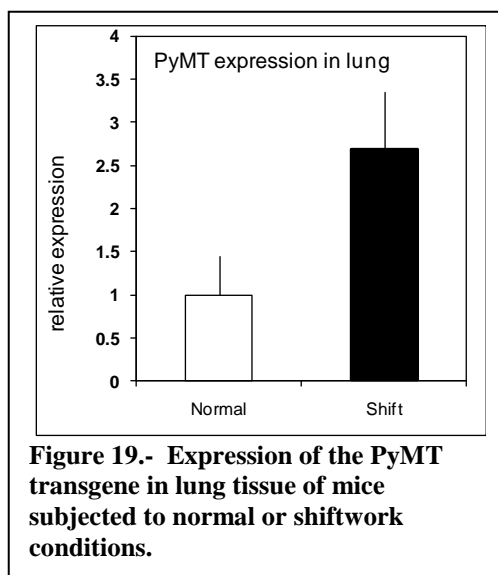
d) Effect of shiftwork on the expression of cell cycle genes in the tumors of PyMT mice .

The expression of *Chk1*, *P21*, *Tgf $\beta$* , *Gadd45a*, *c-Myc* & *P57* mRNA was analysed in the tumors from the PyMT mice. There was no significant rhythm in the expression of any of the genes in the tumors (fig 18). As mentioned in the section dealing with clock gene expression in PyMt tumors, a bimodal pattern was observed in *mTGF $\beta$*  and *m-cMyc* similar to the pattern observed in some of the core clock genes (see fig 16), suggesting a possible interaction amongst these genes. There was a significant increase in the overall expression of the oncogene *m-cMyc* in the animals previously exposed to the shiftwork simulation, compared to the controls under 12L:12D (, 57% increase, graph not shown).



e) Effect of shiftwork simulation on metastasis to the lung in PyMT mice:

In 14-week-old PyMT mice, it was very difficult to distinguish between healthy and cancerous tissue within the mammary gland because the whole gland was hyperplastic. Consequently, it was decided to not attempt to analyse normal mammary tissue from these animals. Instead, the effects of the shiftwork simulation on the degree of metastasis to the lung of these mice were examined. This was accomplished by assessing the expression of the transgenic RNA in the lung. Given that only mammary cells have the capacity to express this transgene, measuring the level of expression of the transgene should correlate with the number of cells that have metastasized to the lungs, thus giving us information on whether the shiftwork simulation affects the degree of metastasis to the lung; a preferential niche of secondary growth of breast cancer cells. Figure 19 shows a significantly higher expression of the PyMT transgene in lungs of mice that had been exposed to the shiftwork simulation ( $P < 0.05$ ). This may reflect a higher number of malignant cells that have metastasized to the lung, and support the contention that shiftwork has a negative impact on the progression of cancer by facilitating the metastasis of breast cancer cells by mechanisms that remain elusive.



**Task 4.- Development of an immunodeficient *Clock*<sup>A19</sup>+MEL mutant mouse strain.**

A line of SCID mice that possess the *Clock*<sup>A19</sup> mutation (Vitaterna et al., 1994) has been established by crossing *Clock*<sup>A19</sup>+MEL mice (Kennaway et al., 2003) with SCID mice. These mice are characterized by peripheral arrhythmicity but conserved central rhythmicity. In an initial attempt to cross *Clock*<sup>A19</sup>+MEL mice with BALB/c-Fox1<sup>nu</sup> mice, the dams had very poorly developed mammary glands and were unable to feed their litters. The pups could, however, survive if cross-fostered to a lactating mother lacking the *Clock*<sup>A19</sup> mutation. This interesting finding suggests that a functional clock is required for normal mammary gland development in this immunocompromised background. Fortunately, when the mutation was crossed into a SCID background, none of these negative effects on mammary gland development were detected.

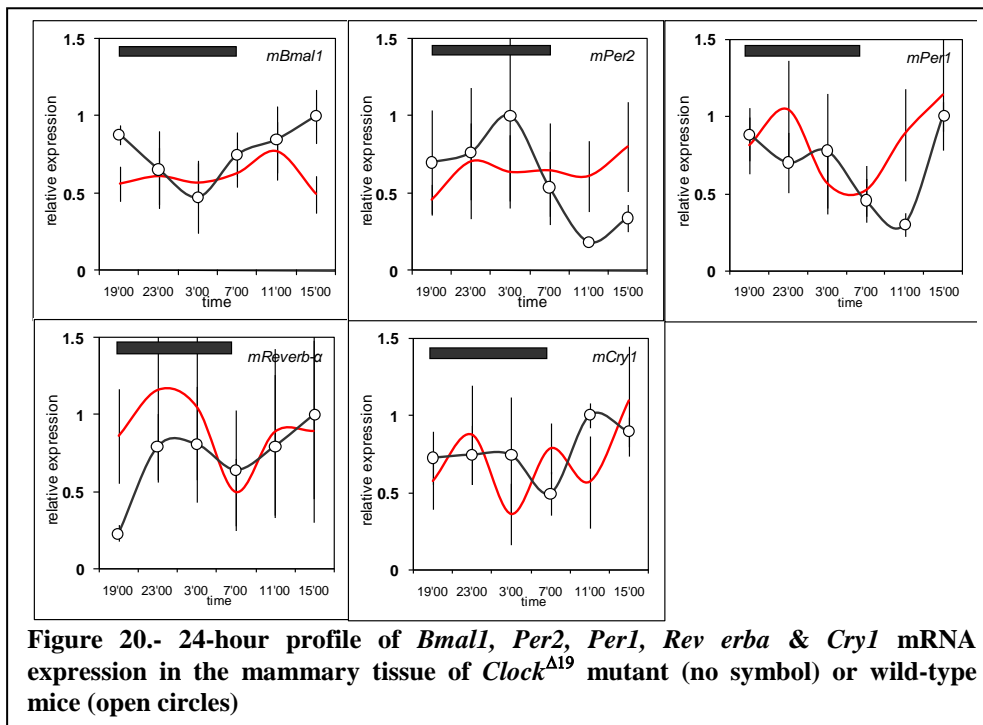
In conclusion, a viable colony of SCID mice which have arrhythmic peripheral tissue clocks was established and was used in the following experiment.

### **Task 5: Growth and gene expression of MCF-7 cells in immunodeficient *Clock*<sup>A19</sup> mutant mice.**

Procedures in this experiment are similar to those described in task 2. Briefly, MCF-7 cells were injected subcutaneously into immunocompromised *Clock*<sup>A19</sup> mutant mice, one week after the implantation of slow release estrogen pellets. The experimental design addressed the role of the host circadian timing system in the regulation of tumor development and growth. As in previous experiments the expression pattern of a suite of core clock genes in the host mammary tissue and also the xenograft were measured in an effort to determine if there is a functional endogenous clock in either of these structures. In addition, the expression of genes involved in the regulation of cell cycle was determined. As previously mentioned, to date we have been able to design human specific primer sets for only *hBmal1*, *hPer2*, *hPer1*, *hCry2*, *hc-Myc*, *hWee1* and *hGadd45a* that do not detect mRNA in the murine stroma that supports the human breast cancer xenografts. This problem is still being addressed outside the current funding.

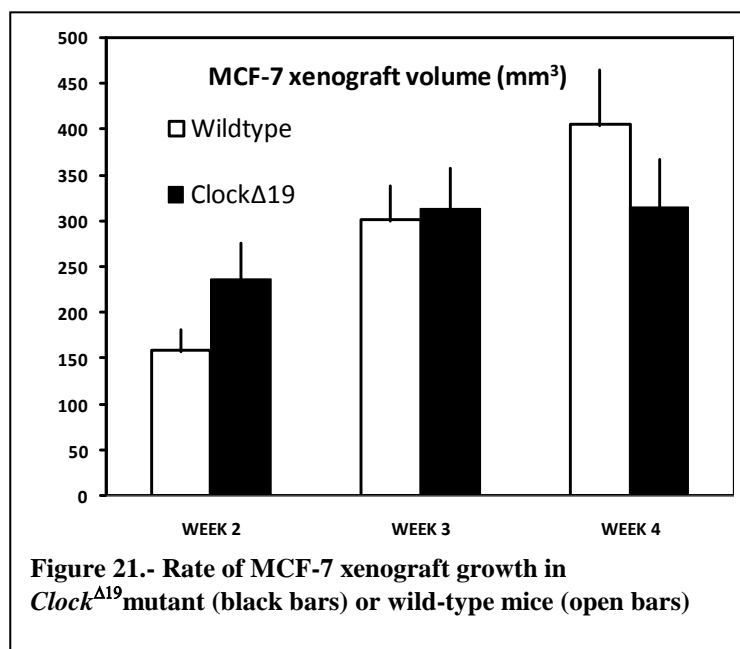
#### **a) Mammary clock gene expression in immunodeficient *Clock*<sup>A19</sup> mutant and wild-type mice**

Wild-type mice expressed *mBmal1*, *mPer2* and *mPer1* mRNA in the mammary gland and the expression of *Bmal1* and the period genes was in antiphase. However, there was considerable variability of the gene expression in the mammary gland and we will further assess liver rhythmicity in these animals to confirm that they have a functional circadian timing system. As expected *Clock*<sup>A19</sup> mutant mice did not show a well defined rhythm in any of the clock genes and the relationship between the genes was lost (fig. 20). These results show that the SCID animals carrying the *Clock*<sup>A19</sup> mutation have a disrupted circadian system.



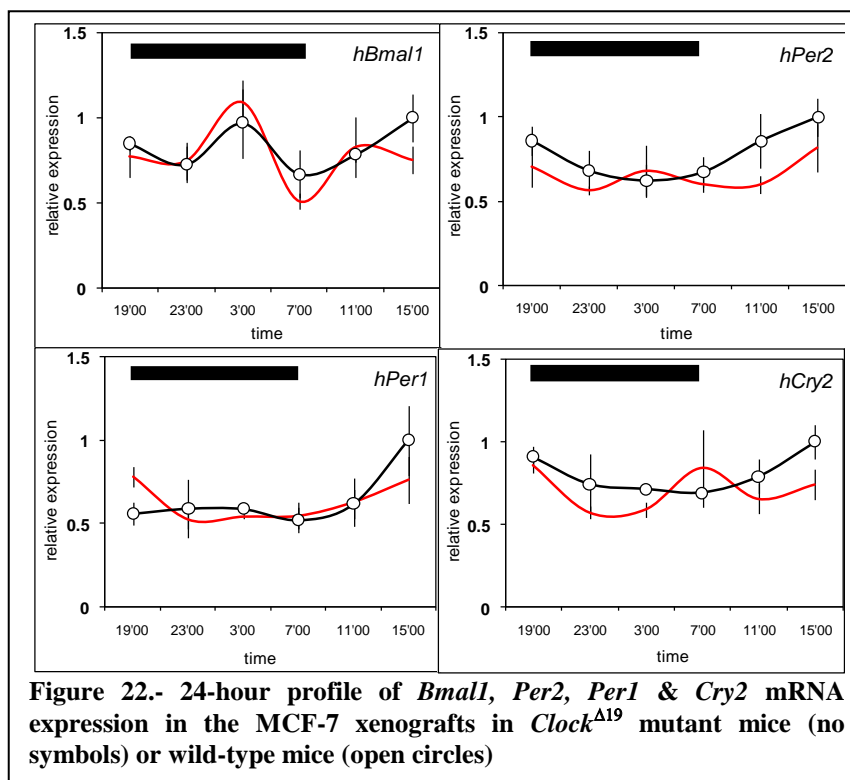
**b) Effect of shiftwork on the rate of growth of MCF-7 xenografts in *Clock*<sup>Δ19</sup> mutant mice**

Innoculation with MCF-7 resulted in the development of tumors in the wild-type mice with the tumor volume doubling between weeks 2 and 4 after injection (fig. 21). Tumors also developed in the *Clock*<sup>Δ19</sup> mutant mice but the rate of growth was different from the wild-type mice such that there was a significant time X treatment interaction. These results provide good preliminary evidence of a potential supportive role of the host's circadian time-keeping system in the regulation of tumor growth.



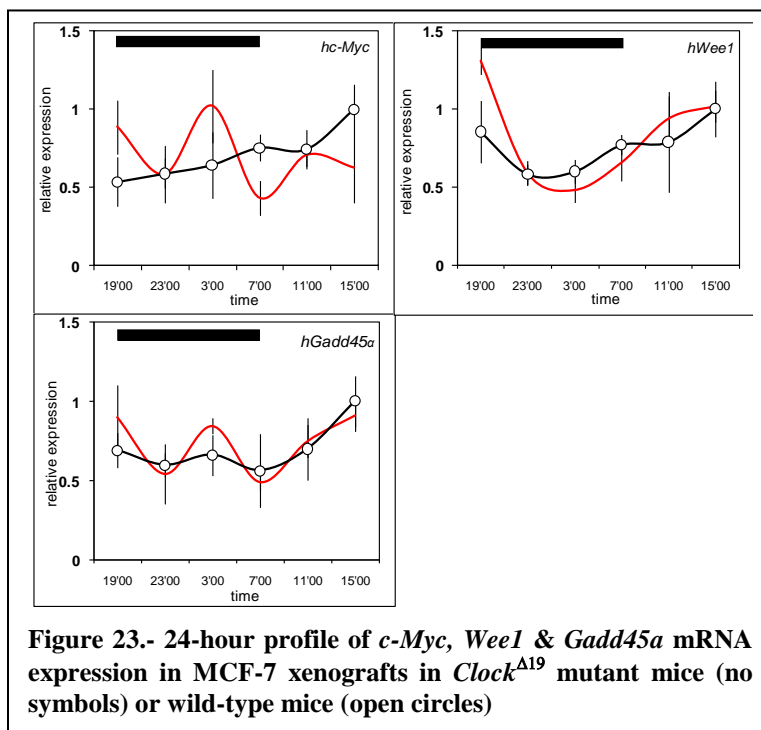
### c) Effect of shiftwork on the expression clock genes in the MCF-7 xenograft

The expression profile of *hBmal1*, *hPer2*, *hPer1* and *hCry2* was determined in the tumors (fig. 22). None of the genes showed a significant rhythm by Cosinor Analysis and the phase relationship of the peaks did not display a pattern that supports a functional circadian timing system in these tumors in experimental groups. Interestingly, the lack of a functional timing system in the host did not have a significant effect on the global level of expression of the core clock genes in these tumors.



d) Effect of shiftwork on the expression of cell cycle genes in MCF-7 xenografts.

When the expression of *c-myc*, *wee1* and *gadd45a* was analysed in MCF-7 xenografts there was no circadian rhythm of expression of these genes (fig. 23). Furthermore the overall expression of these genes was not affected by the lack of a functional circadian clock in the host animal.



**Task 6.- Effect of disruption of clock gene function with small interfering mRNA on the growth and gene expression of human breast cancer cell lines *in vitro* and *in vivo***

*hBmal1* expression has been successfully knocked down in MCF-7 cells and these have been clonally selected and implanted into SCID mice.

Tumors, liver and mammary tissue have been collected 4-hourly over a period of 24 hours as described above and will be analysed in the coming weeks. The information derived from this task will be submitted as a supplement to this final report in coming weeks.

## **KEY RESEARCH ACCOMPLISHMENTS**

**Task 1:** Completed, manuscript in preparation

**Task 2:** Task has been completed. Manuscript in preparation. Some technical difficulties arose in designing human specific primers that do not detect the mouse mRNA.

**Task 3:** Task has been completed. Manuscript in preparation. We additionally examined the impact of shiftwork on the formation of secondary tumors to the lung.

**Task 4:** A viable *SCID*+*Clock*<sup>A19</sup> mutant mouse colony has been established.

**Task 5:** This task has been completed and we are in the process of preparing a manuscript.

**Task 6:** We have created a clock-deficient MCF-7 clonal line with knocked down *mBmal1* using shRNA. These cells have been injected into SCID mice and the resulting tumors excised after 4 weeks. Tissue sampling is completed and we are in the process of assessing and analyzing the data. The information derived from these experiments will be submitted as a supplement to this document.

## REPORTABLE OUTCOMES.

- 1.- Four weeks of simulated shiftwork resulted in the development of abnormal relationships between gene expression rhythms, the light/dark cycle and behavior in the mouse liver and mammary glands. One day after returning to the original photoperiod, the expression of clock gene transcription factors was approximately 180 degrees out of phase with expression in animals that had been kept on a stable 12L:12D photoperiod. There was a similar inversion in the timing of the rhythms of expression of the cell cycle genes *mP21* and *mWee1* in the mammary glands. Rhythmicity of *mchk1* mRNA was lost 24 hours after the last change. Interestingly there was a down-regulation of *mPer2* mRNA and up-regulation of *mTim* mRNA expression following exposure to the shiftwork simulation.

The implications of these results have the potential to affect the normal development of cell cycle on several fronts. The shift in peak of expression of the cell cycle genes is likely to impinge on the time of day that cell division occurs. Indeed, it may be important to restrict cell division to time of the day when there is a low exposure to genotoxic stresses such as UV light, electromagnetic radiation or other putative carcinogens. In shiftworkers, cell division may occur when these people are exposed to some of these negative environmental factors and thus increase the risk of cancer. On the other hand, the loss of rhythmicity of genes such as *mChk1* may have a negative impact at times of the day when this cell cycle checkpoint is not expressed at adequate levels to mediate the impact of environmental genotoxic challenges. Finally, the downregulation of *mPer2* is likely to have major negative impact on the incidence of cancer. Although the mechanisms still remain elusive it is well accepted that in addition to its role as a transcription factor in the circadian timing machinery, *mPer2* also has the capacity to act as a tumour suppressor gene. Consequently, downregulation of this gene in the mammary gland is likely to have direct negative consequences on the incidence of breast cancer in female shiftworkers.

- 2.- When MCF-7 cells were injected into mice that had been in the shiftwork simulation for 4 weeks prior to inoculation then maintained under these conditions for a further 4 weeks, the rate of tumour growth was not different from that in the controls. Expression of clock genes in the normal mammary tissue of the mice that had been subjected to a total of 8 weeks of shiftwork simulation was severely affected. Expression of *mBmal1*, *mPer1*, *mRev erba*, *mCry1* and *mCry2* were all downregulated.

In regards to the pattern of gene expression in the xenograft, the expression pattern of the core clock genes suggested there was not a functional circadian clock in the cells that made up the tumour. We also examined two cell cycle genes that did not display any remarkable changes. Given that *Wee1* is known to have a response element to the CLOCK:BMAL1 dimer in its regulatory region, it is not reasonable to expect a rhythmic pattern of expression, given the lack of a functional circadian timing system in these cells. It is possible that, the cues supplied by the host were not capable of synchronizing the rhythms of each individual cell comprising the tumour; a situation that is compatible with the results we are showing. Finally, shiftwork did not have an impact on the rate of growth of the xenograft.

- 3.- When PyMT oncogene transgenic mice were subjected to the shiftwork simulation from weaning to 14 weeks of age, the total tumour burden was significantly higher in the treated mice. There was no circadian rhythm of expression of core-clock genes or cell-cycle genes in

the tumour derived from these animals although rhythmicity was present in the livers of the animals. Interestingly, we detected a 2.7 fold higher expression of PyMT transgene in the lungs of the treated mice, which provides strong evidence of increased metastasis to the lung.

**4.-** A new animal model was established following the crossing of SCID mice with *Clock*<sup>Δ19</sup> mutant mice. These mice lack rhythmic gene expression in their peripheral tissues and are immunocompromised, which allowed us to study the effects of host rhythmicity on xenograft growth and function.

**5.-** MCF-7 xenografts grow more slowly in *Clock*<sup>Δ19</sup>/SCID mice. The pattern of gene expression in these tumours was quite unremarkable. No circadian rhythmicity was detected using Cosinor Analysis in core-clock or cell-cycle genes. The global level of gene expression in the xenografts was unchanged for all the genes we examined.

**6.-** We have created a clonal line of clock-deficient MCF-7 breast cancer cells that in which *hBmal1* has been knocked down using shRNA.

## CONCLUSIONS

1.- Exposing mice to simulated shiftwork alters and/or disrupts the circadian expression of clock gene transcription factors and genes that are involved in the regulation of the cell cycle. This provides a molecular mechanism through which shiftwork has a clear impact on the dynamics of cell division and apoptosis.

2.- Exposing mice to simulated shiftwork altered the global level of expression of several clock and cell-cycle genes, including an important tumour suppressor gene such as *Per2*. That shiftwork is capable of affecting the level of expression tumour suppressor genes provides another clear mechanism that explains the negative impact of shiftwork on the genesis and progression of cancer.

3.- The SSW used in this project favored the formation of lung metastasis.

4.- MCF-7 xenografts grow slower in mice with a deficient circadian timing system.

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